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(54) Title: GENE SWITCH

(57) Abstract

A gene promoter sequence which responds to application of an exogenous chemical inducer such as one of the plant herbicide safeners is utilised as a gene switch to enable external control of expression of a gene placed under control of the promoter. A specific example of the gene switch construct is the maize GSTII (glutathione-S-transferase isoform II) enzyme promoter sequence which is induced by external application of the safener N,N-diallyl-2,2-dichloroacetamide.

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GENE SWITCH

This invention relates to a gene promoter sequence which is responsive to an applied exogenous chemical inducer enabling external control of expression of the gene controlled by the promoter. The sequence of the invention is conveniently referred to as a gene switch.

European Patent Application 332,104 (published 18th September 1989) describes chemically regulatable DNA sequences which are capable of regulating transcription of an associated DNA sequence in plants or plant tissue. The particular source of the sequences is described as being the PR (pathogenesis-related) protein gene.

According to the invention there is provided a chemically inducible gene promoter sequence isolated from a 27kd subunit of the maize glutathione-S-transferase (GST II) gene.

The invention also provides a chemically switchable gene constructs which include the maize GST II gene promoter operatively linked to a foreign gene or a series of foreign genes whereby expression of said foreign gene or said series of genes may be controlled by application of an effective exogenous inducer.

In practice the chemically inducible promoter of the invention will be inserted as a promoter sequence in a recombinant gene construct destined for use in a plant. The construct will then be

inserted into the plant by transformation. Expression of protein encoding genes in the construct, being under control of the chemically switchable promoter of the invention, may be controlled by the application of a chemical inducer to the plant.

The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using Agrobacterium tumefaciens or its Ti plasmid, electroporation, microinjection of plant cells and protoplasts, microprojectile transformation and pollen tube transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

Neither is the plant species into which the chemically inducible promoter is inserted particularly germane to the invention. Dicotyledonous plant are rel;atively simple to transform whereas the monocotyledonous plants (which include the major cereal crops such as maize) are rather more difficult to transform. However, this invention may be applied to any plant for which transformation techniques are, or become, available.

Therefore, the invention further provides a plant having a recombinant gene construct which includes the chemically inducible promoter of the invention stably incorporated in its genome by transformation.

The invention also provides an effective promoter/inducer combination wherein the promoter is the GST II promoter aforesaid and the inducer is N,N-diallyl-2,2-dichloroacetamide (common name: dichloramid) or benzyl-2-chloro-4-(trifluoromethyl) -5-thiazole-carboxylate (common name: flurazole)

However, more generically, the present invention comprises a recombinant DNA construct including a gene promoter inducible by application of an exogenous chemical inducer operatively linked to control expression of a target gene. Suitable promoters and specific inducers may be identified by the procedures exemplified hereinbelow in respect of the GST II gene promoter and its inducer. In other words, the invention comprises the use of a gene promoter which is inducible by externally applied chemical to control expression of a gene sequence within a plant genome.

Chemical inducers which are potential inducers of the GSTII 27kd subunit expression include compounds such as:

1. benzyl-2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate;
2. naphthalene-1,8-dicarboxylic anhydride;
3. 2-dichloromethyl-2-methyl-1,3-dioxolane;
4. 1-(dichloroacetyl)-hexahydro-3,3,8a-trimethyl-pyrrole (1,2-a)-pyrimidin-6(2H)-one;
5. 2,2,5-trimethyl-N-dichloroacetyloxazolidine;
6. 1,3-dioxolan-2-ylmethoxyimono(phenyl)benzene acetonitrile;
7. 4,6-dichloro-2-phenyl-pyrimidine;
8. 2,2-dichloro-[N-allyl-N(1,3-dioxalano-2-methy)] acetamide;
9. 1-(cyanomethoxyimino)benzacetonitrile;

10. 4'-chloro-2,2,2-trifluoroacetophenone-O-1,3-dioxolan- 2-yl methyloxime;
11. 2,2-dichloro-1-(3,4-dihydro-3-methyl-2H-1,4-benzoxazin-4-yl) ethanone;
12. 3-dichloroacetyl-2,2-dimethyloxazolidine;
13. 4-methoxy-3,3-dimethylbenzophenone;
14. 1-cyclohexyl-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pent-1-en-3-ol;
15. 2,2-dichloro-N-(3-methyl-4-thiazolin-2-ylidene) acetamide;
16. O,O-diethyl-O-phenyl phosphorothioate;
17. 2,2-spirocyclohexyl-N-dichloroacetyl oxazolidine;
18. N-benzyl-N-ethyl-dichloroacetamide;
19. 3-chloroacetyl-4,4-cyclohexane-spiro-2,2-dimethyl-1,3- oxazolidine; and,
20. spirooxazolidine acetamide.

Glutathione-S-transferases (GST) are a family of enzymes which catalyse the conjugation of glutathione via the sulphydryl group to a large range of hydrophobic, electrophilic compounds. The conjugation results in detoxification of these compounds and in insects and mammals, removal from tissue.

GST enzymes have been identified in a range of crop plants including maize, wheat, sorghum and peas. GST's comprise from 1 to 2% of the total soluble protein in etiolated maize seedlings.

The major isoform of GST can be distinguished in maize tissue. GST I is constitutively expressed and is capable of conjugating glutathione with the pre-emergent herbicides alachlor and atrazine. Treatment of maize tissues with chemical safeners (for example, N,N-diallyl-2,2-dichloroacetamide)

raises the activity of GST I which participates in the detoxification of the pre-emergent herbicides.

The invention will now be described by way of illustration in the following description.

The drawings which accompany this application are as follows:

Figure 1 Figure 1 shows the results for total GST activity in roots and shoots obtained 23 and 44 hours after treatment with R25 as described below;

Figure 2 shows the chromatographic separation of the isozymes GST I and GST II.

Figure 3 shows GST I activity present in untreated anther tissue;

Figure 4 shows the stimulation of GST II activity after treatment with R25 as described below;

Figure 5 shows the results using a stem reservoir technique;

Figure 6 shows the results with the application by spray; and,

Figure 7 is a time course graph generated in the manner described below.

Safener treatment of corn tissue

For treatment of young maize seedlings, seeds were germinated on moist filter paper. After germination and growth (up to one week) the safener N,N-diallyl-2,2- dichloroacetamide (hereinafter referred to as R25) was added to the water in the filter paper to give a range of concentrations (0.003 to 30 ppm) and the seedlings grown for a further 23 to 44 hours before harvesting of root and shoot tissue. Figure 1 shows the results for total GST activity in roots and shoots obtained 23

and 44 hours after treatment as described and Figure 2 shows the separation of the isozymes GST I and GST II.

For treatment of maize tassel and anther tissue, a solution of 800 μg of R25 was injected into the node directly below the developing tassel. Uptake then continued for a further 48 to 72 hours. Figure 3 shows that only GST I activity was present in untreated anther tissue and Figure 4 shows the stimulation of GST II activity after treatment as described.

Alternatively a 100 ppm solution of R25 was supplied from a glass reservoir attached to the exposed stem immediately below the developing tassel. Figure 5 shows the results using a stem reservoir technique.

Additionally, R25 was applied as a 100 ppm spray directly on to the exposed developing tassel. Figure 6 shows the results with the application by spray.

Both GST proteins have a native molecular weight of approximately 50 kd. As in mammals, maize GST's are dimeric; GST I has apparently identical subunits of 29 kd, whereas GST II is a heterodimer of a 29 kd subunit similar to that found in GST I and a novel 27 kd subunit which is only present in tissue treated with safener except in seedling root where it is constitutively expressed, but still can be induced by safener treatment.

A cDNA and a gene corresponding to the 29 kd subunit of GST I have been cloned previously and sequenced. In addition, a cDNA corresponding to a 26 kd subunit of a third, minor component of GST

activity in maize seedlings (GST III) has been previously cloned and sequenced.

Enzyme Assay

Enzyme activity was measured spectrophotometrically at 340nm using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The reaction buffer contained 0.1M EDTA, 0.001M CDNB and 0.0025M glutathione.

Preparation of extracts and enzyme purification

Tissue was homogenised in 0.05M Tris.HCl, pH 7.8; 0.001M EDTA; 0.001M DTT; and 7.5% polyvinylpyrrolidone in a pestle and mortar, at 4°C, and centrifuged at 30,000g to obtain a crude extract.

Separation of the GST isoforms from the crude extract was achieved as follows: the crude extract was applied to a DEAE Sepharose column and washed with 0.01M Tris.HCl, pH 7.8; 0.001M EDTA; and 0.001M DTT. The bound GST was eluted with 0.3M potassium chloride. Fractions containing GST activity were combined and desalted using PD10 gel filtration columns. Separation of the GST I and GST II isoforms was achieved by FPLC on a mono-Q column and a zero to 0.4M potassium chloride concentration gradient.

Pure samples of GST I and GST II were obtained by applying desalted fractions of GST I and GST II from the FPLC to a glutathione-S-sepharose affinity column equilibrated with 0.05M phosphate buffer at pH 7.3. After washing with buffer, bound GST was eluted with 0.005M glutathione.

SDS-PAGE (17.5%, 30:0.174 acrylamide: bisacrylamide) of GST I or GST II was achived by concentrating pure GST samples using Amicon

Centricon 10 Microconcentrations (Trade Mark), denaturing samples in mercaptoethanol containing Laemmli buffer, and staining the gels with Coomassie Blue.

Generation of antibodies to the enzyme

Sufficient protein to enable the immunisation of rabbits is obtained by pooling the isolated enzyme subunit isolated as described above from a number of separate experiments. The 27 kD GST II polypeptide is subsequently purified to apparent homogeneity by electroelution from polyacrylamide gel slices. Antisera are prepared against the 27 kd polypeptide. The immunisation of rabbits is carried out essentially according to Mayer and Walker (1978).

N-terminal sequence analysis

The amino terminal sequence of the intact 27 kd subunit of GST II or partial proteolytic cleavage products was determined by sequential Edman degradation and subsequent amino acid analysis by HPLC.

Time Course

A time course experiment was carried out to examine the expression of GST's after safener treatment. A 30 ppm solution of R25 was applied to three-day old seedling roots and tissue harvested after various time intervals following safener treatment. Samples were tested for GST activity using the enzyme assay described above. The results of this experiment are presented graphically in Figure 7.

Synthesis of cDNA libraries

The time course experiments revealed a peak of GST expression at 48 hours after treatement with

safener. Therefore, two cDNA libraries were constructed from RNA extracted from tissue at 24 and 48 hours after safener treatment. To ensure that the induction procedure had been successful, a one gram sample of 24 hour induced tissue was taken and assayed for GST II. This experiment revealed that the tissue used to construct the cDNA library had indeed been successfuly induced as GST II accounted for 45.5% of the total GST activity.

Double-stranded cDNA was prepared from oligo dT-cellulose-purified RNA by a method employing RNaseH and E.coli DNA polymerase I in the synthesis of the second strand, without prior purification of single-stranded cDNA (Gubler and Hoffman,1983).

Screening cDNA libraries with antisera to GST I and GST II

In order to identify a cDNA clone encoding maize tassel GST enzyme, bacteriophage from the amplified cDNA library are screened with anti-maize GST enzyme serum. The clones producing the strongest signals are re-screened.

Screening cDNA libraries using oligo probes

Mixtures of synthetic oligonucleotides based on the amino acid sequence determined above were prepared by phosphoramidite chemical synthesis. The 5′ ends of the oligonucleotides were labelled using polynucleotide kinase as described in the literature.

Approximately 40,000 phages containing cDNA were amplified on plates and transferred to nitrocellulose. The filters were hybridised to oligonucleotide probes at temperatures of from 2 to 5°C below the melting temperature calculated for

the lowest melting point probe in the mixture. Hybridising plaques were selected and rescreened through two or more rounds exactly as described above but at lower densities

Isolation of cDNA gene sequences by the PCR method

cDNA or DNA sequences are isolated from the libraries described using oligo primers based on the amino acid sequence obtained from partial proteolytic cleavage or in the case of genomic DNA, primers based on cDNA sequence determined previously.

Characterisation and sequence analysis of GST cDNA clones

The isolated cDNA is characterised and subjected to sequencing by one or more of the standard available techniques.

Isolation of genomic sequences

An existing genomic library of fragments of total maize DNA cloned into λEMBL3 is used to isolate clones that hybridise to the cDNA clones isolated as described above.

Alternatively, the PCR method described above may be used to selectively amplify and clone gene fragments. GSTII genes and ther promoter sequences can then be isolated, and characterised using established techniques. It can be demonstrated that the GSTII promoter sequences mediate safener-induced gene activity by fusing them to marker genes like GUS and CAT, and testing then in transgenic plants.

CLAIMS

1. A chemically inducible gene promoter sequence isolated from a 27kd subunit of the maize glutathione-S-transferase (GST II) gene.

2. A chemically switchable gene construct which includes the maize GST II gene promoter operatively linked to a gene or a series of genes whereby expression of said gene or said series of genes may be controlled by application of an effective exogenous inducer.

3. A plant having a construct as claimed in claim 2 stably incorporated in its genome by transformation.

4. A promoter/inducer combination wherein the promoter is the GST II promoter aforesaid and the inducer is N,N-diallyl-2,2-dichloro-acetamide or benzyl-2-chloro-4-(trifluoro-methyl)-5-thiazole-carboxylate.

5. A promoter/inducer combination wherein the promoter is the GST II promoter aforesaid and the inducer is N,N-diallyl-2,2-dichloro-acetamide.

FIG. 1.

Stimulation of GST activity in roots and shoots by safener treatment

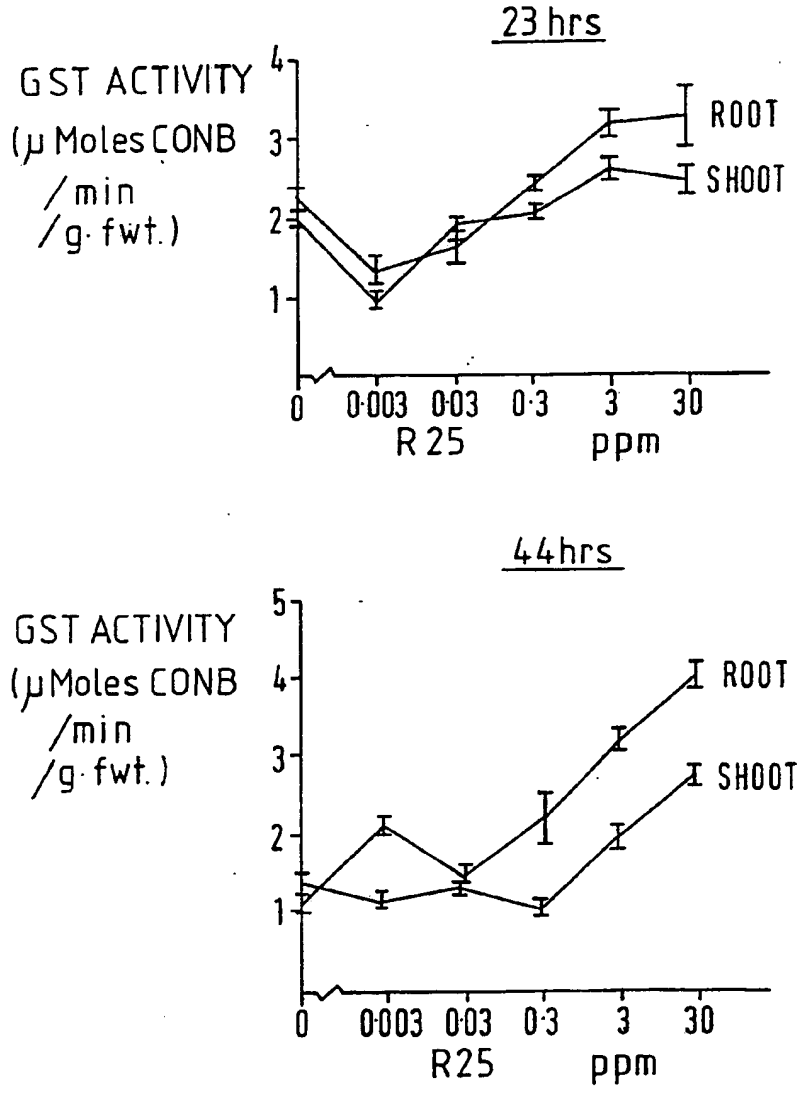
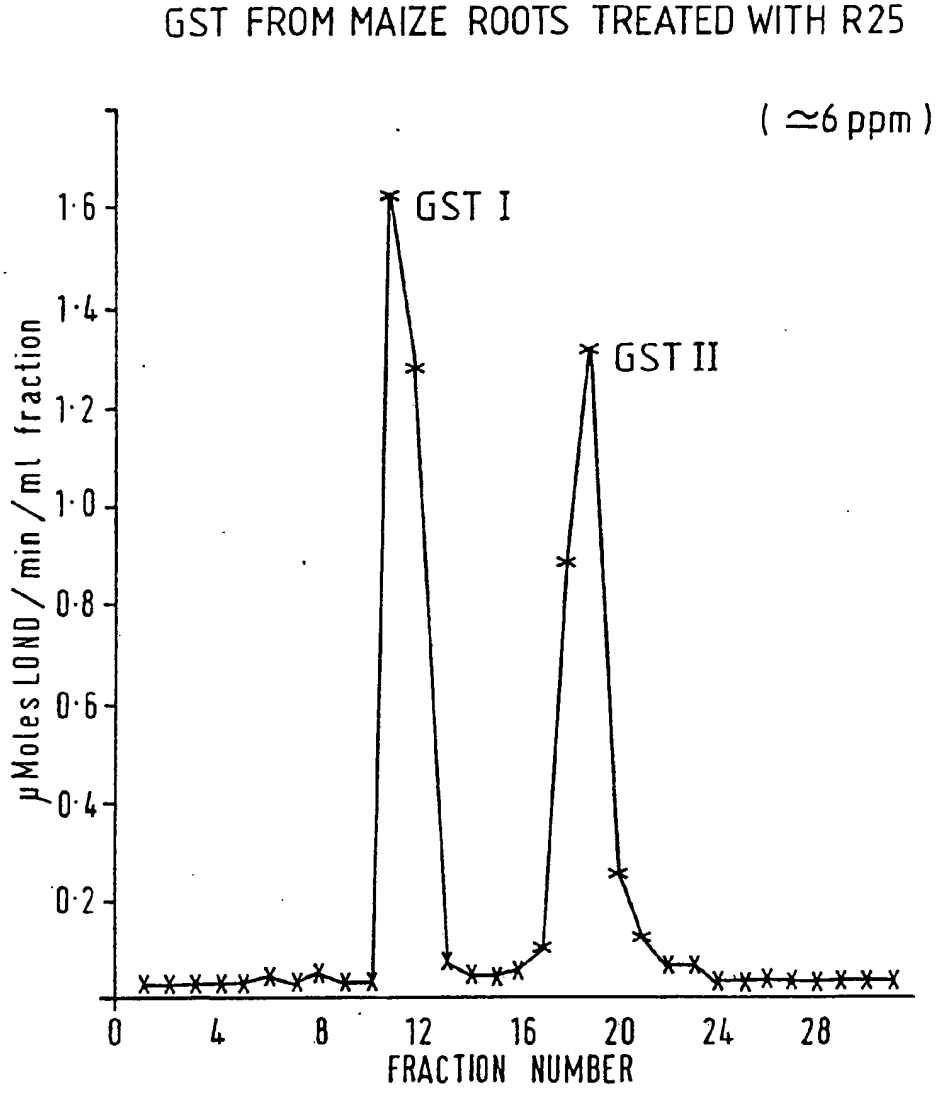


FIG. 2.

Separation of GST I and GST II in roots tissues treated with R25



GST I ≃ 0·9% OF TOTAL SOL PROTFIN

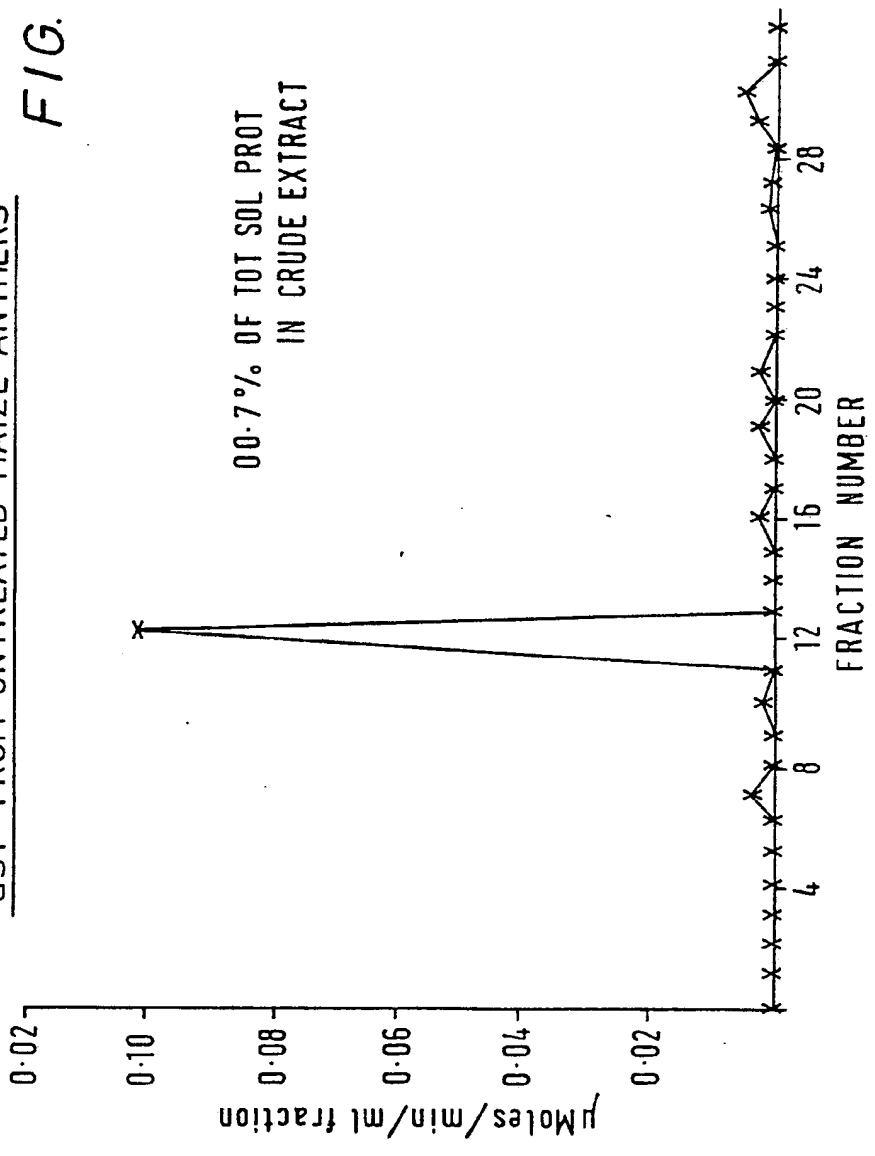
GST II ≃ 2·5% OF TOTAL SOL PROTFIN

[USING DATA FROM MOZER (MONSANTO)]

GST I is the only isoform present in untreated anthers

GST FROM UNTREATED MAIZE ANTHERS

FIG. 3.



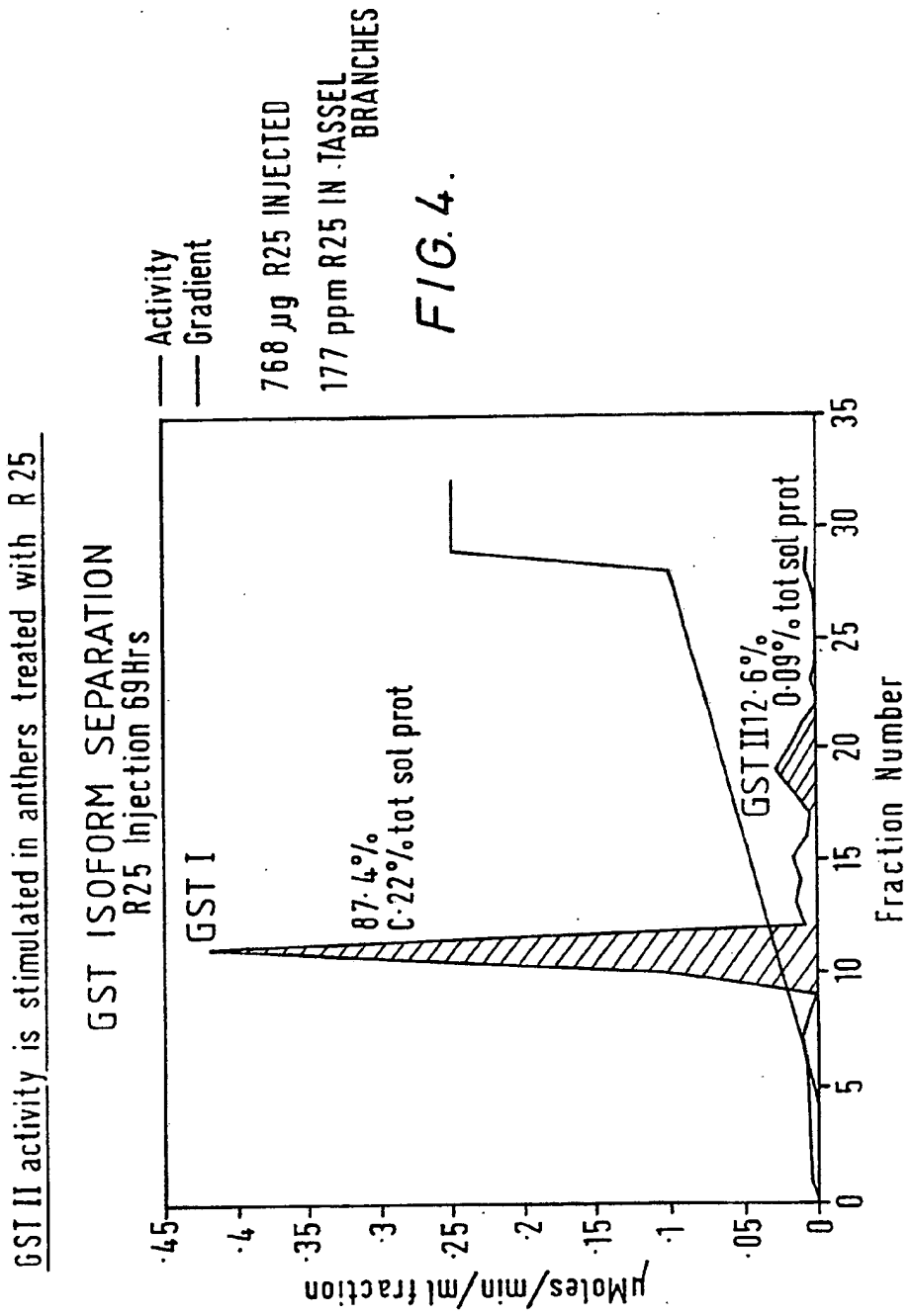

GST II activity is stimulated in anthers treated with R 25
GST ISOFORM SEPARATION
R25 Injection 69Hrs
Activity
Gradient
768 µg R25 INJECTED
177 ppm R25 IN TASSEL BRANCHES
GST I
87·4%
C·22% tot sol prot
GST II 12·6%
0·09% tot sol prot
µMoles/min/ml fraction
Fraction Number
·45
·4
·35
·3
·25
·2
·15
·1
·05
0
5
10
15
20
25
30
35

FIG. 4.

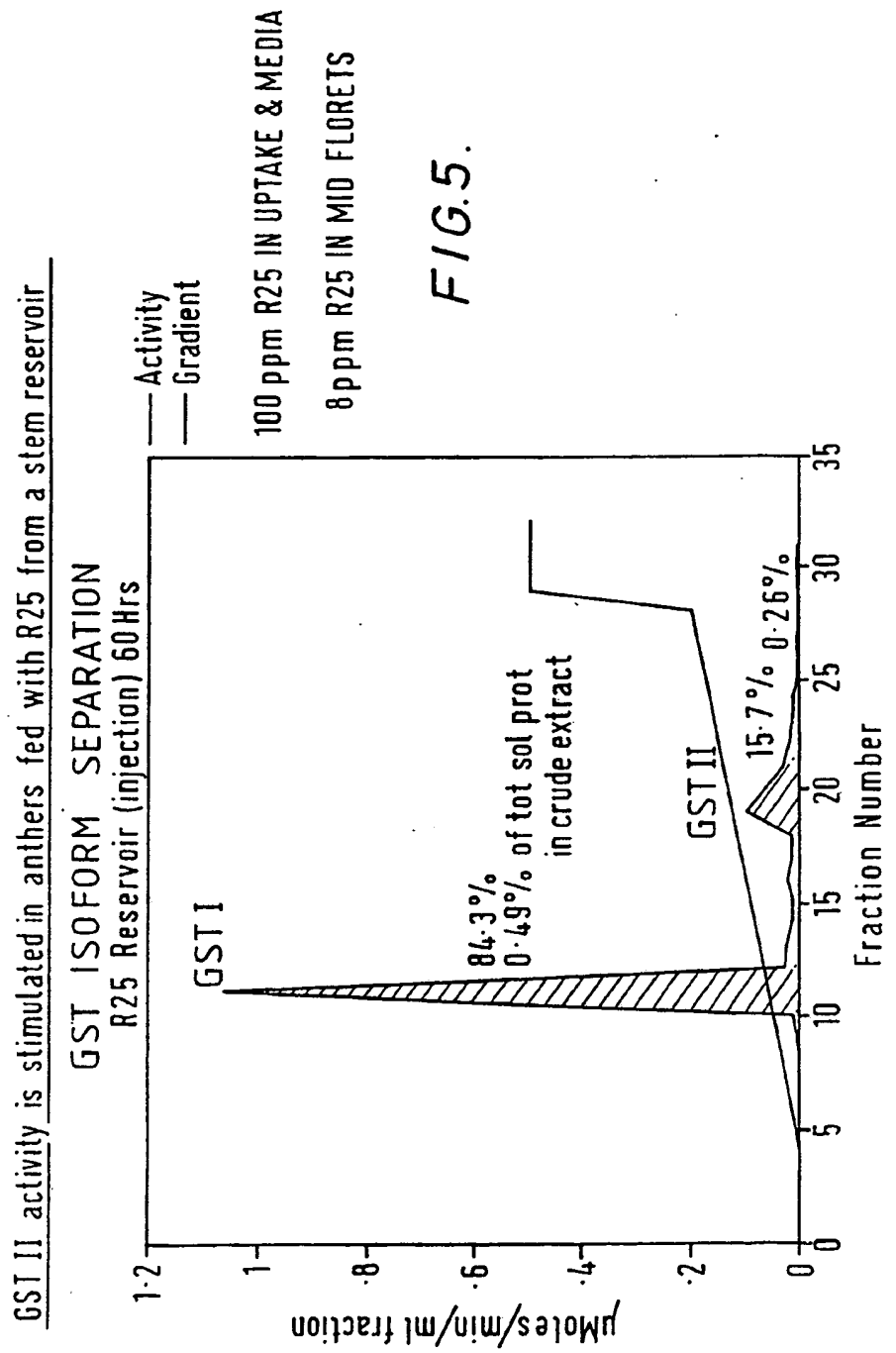

GST II activity is stimulated in anthers fed with R25 from a stem reservoir
GST ISOFORM SEPARATION
R25 Reservoir (injection) 60 Hrs
Activity
Gradient
100 ppm R25 IN UPTAKE & MEDIA
8 ppm R25 IN MID FLORETS
GST I
84·3%
0·49% of tot sol prot
in crude extract
GST II
15·7% 0·26%
μMole s/min/ml fraction
Fraction Number
1·2
1
·8
·6
·4
·2
0
0
5
10
15
20
25
30
35

FIG.5.

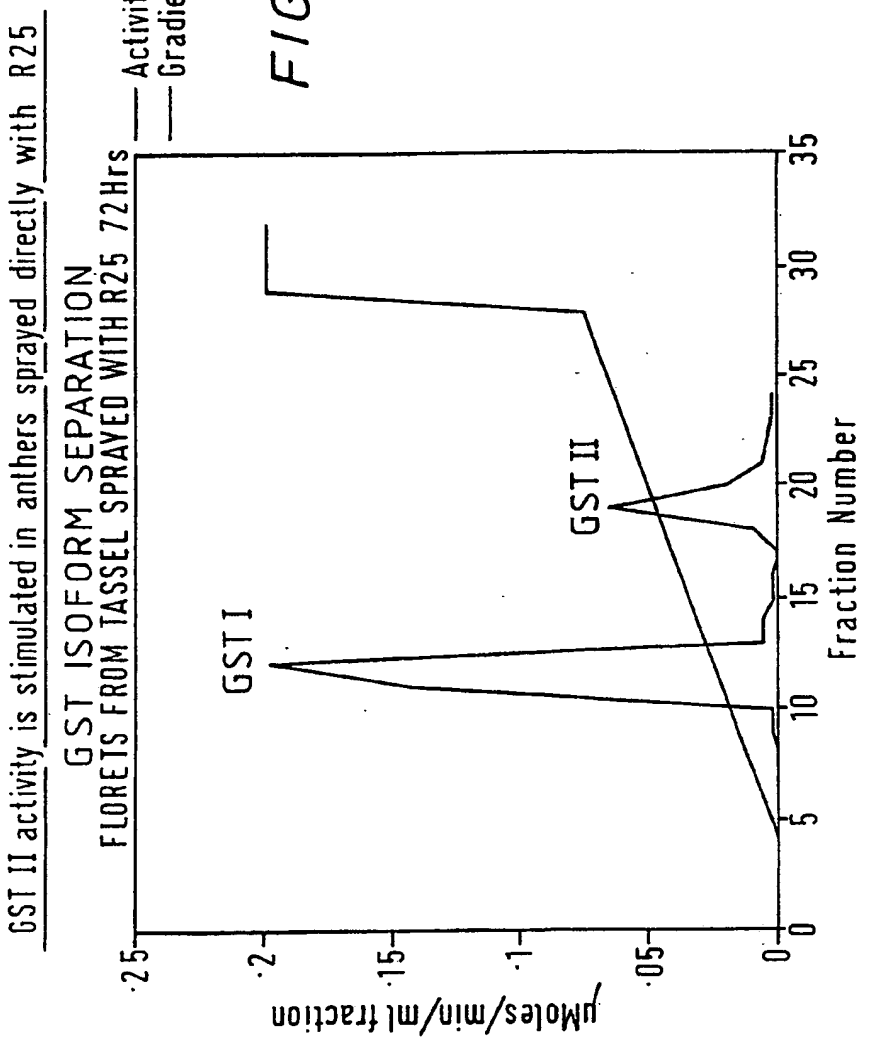
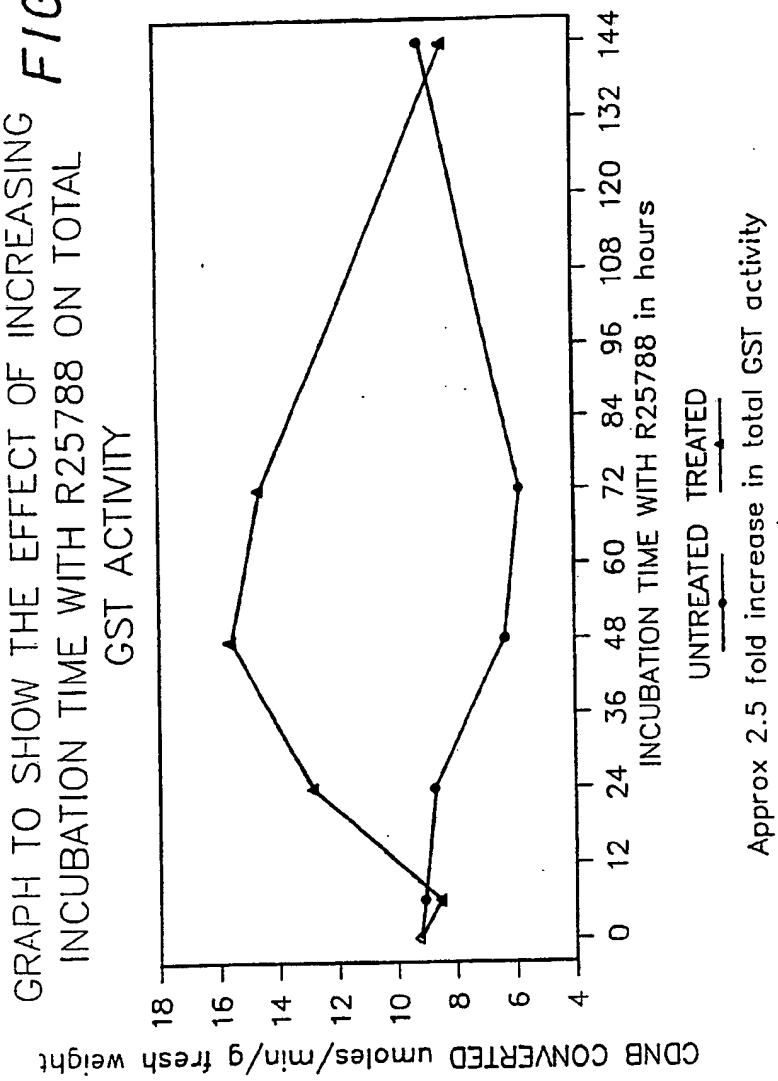

GST II activity is stimulated in anthers sprayed directly with R25
GST ISOFORM SEPARATION
FLORETS FROM TASSEL SPRAYED WITH R25 72Hrs
Activity
Gradient
GST I
GST II
μMoles/min/ml fraction
Fraction Number
·25
·2
·15
·1
·05
0
5
10
15
20
25
30
35

FIG. 6.

FIG. 7.

GRAPH TO SHOW THE EFFECT OF INCREASING INCUBATION TIME WITH R25788 ON TOTAL GST ACTIVITY



Approx 2.5 fold increase in total GST activity at 48 hours.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/00101

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/54, C 12 N 15/82

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Plant Molecular Biology, volume 7, 1986, Martinus Nijhoff Publishers, (Dordrecht, NL), R.C. Wiegand et al.: "Messenger RNA encoding a glutathione-S-transferase responsible for herbicide tolerance in maize is induced in response to safener treatment", pages 235-243 see the whole article --	1-5
A	Biochemistry, volume 22, no. 5, 1983, American Chemical Society, T.J. Mozer et al.: "Purification and characterization of corn glutathione S-transferase", pages 1068-1072 see the whole article --	1-5
A	Chemical Abstracts, volume 104, 1986, (Columbus, Ohio, US), D.M. Shah et al.: "Structural analysis of a maize gene coding for glutathione-S-transferase ./.	1-5

* Special categories of cited documents: [10]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th April 1990	11. 06. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

International Application No. PCT/GB 90/00101

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	involved in herbicide detoxification", see page 147, abstract 180998d, & Plant Mol. Biol. 1986, 6(4), 203-11 --	
A	Nature, volume 335, 29 September 1988, W.R. Marcotte Jr et al.: "Regulation of a wheat promoter by abscisic acid in rice protoplasts", pages 454-457 see the whole article --	1-5
O,A	Journal of Cellular Biochemistry, Supplement 12C, The Molecular Basis of Plant Development, 26 March - 2 April 1988, Alan R. Liss, Inc., (New York, US), R.S. Quatrano et al.: "Control of cereal embryogeneses and the regulation of gene expression by abscisic acid (ABA)", page 152, abstract L 052 see the abstract	1-5
